AUTOMATED CALCIUM MEASUREMENTS IN LIVE CARDIOMYOCYTES

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ABSTRACT

Heart failure due to hypertension, infarction, or other factors, is a leading killer of men and women in modern society and involves, at its base, a debilitating loss of cardiomyocytes. Recent studies indicate the feasibility of regenerating lost cardiomyocytes by transplanting embryonic stem cell-derived cardiomyocytes (ESCMs) or mobilizing resident stem cells. To realize the potential of stem-cell based therapies, we hypothesize that it will be extremely beneficial to develop technology and instruments for high throughput, high content screening (HCS) of drugs and genes for their ability to stimulate the formation of functional, contractile cardiomyocytes. Contractile activity is the primary physiological function of cardiomyocytes and abnormal contractility is potentially lethal. We discuss the first phase of the development of an instrument dedicated to distinguishing differentiated ESCMs from undifferentiated non-cardiac background cells using automated cell-by-cell quantification of contractile-calcium transients as the primary assay.

INTRODUCTION

The contractile cycle of cardiomyocytes (Figure 1) is divided into an abrupt shortening phase (systole), induced by a rapid rise in the intracellular calcium concentration ($[Ca^{2+}]_i$) due to calcium entry via voltage dependent L-type calcium channels and calcium-induced-calcium release from the sarcoplasmic reticulum (SR). Contraction is followed by relaxation of the cell (diastole) and the decline of $[Ca^{2+}]_i$. Decline of $[Ca^{2+}]_i$during diastole is controlled primarily by re-sequestration of calcium back into the SR by SERCA2, an ATPase associated with the SR membrane.

Calcium transients have been recorded from cardiomyocytes derived from embryonic stem cells (both from mouse and from human) by several laboratories. For example, calcium transients in fura-2-loaded cardiomyocytes derived from human embryonic stem cells (hESC-CM), were observed in spontaneously contracting embryoid bodies [2]. hESC-CMs cultured on mouse visceral-endoderm like cells showed similar calcium transients and spontaneous contractions at 0.6 to 1.5 Hz [3]. Fluo-4 has been used to record calcium transients from murine ESCMs, which exhibited spontaneous contraction rates of approximately 1 Hz [4]. Consistent with the wide spread observation of spontaneous beating in experiments designed to elicit the appearance of ESCMs, myocytes with "pace-maker" activity have been observed in contracting cell clusters, serving to drive the contractions of neighboring cells [5,6]. These studies indicate that ESCMs typically exhibit calcium transients and contractile characteristics similar to neonatal rat ventricular myocytes (NRVMs), rather than adult ventricular cardiomyocytes. Thus, for our experimentation we used NRVMs as a test-bed to develop our instrument.

Automated high content screens allow for a wealth of information to be gathered from a given experimental study. If the hardware and controlling software are present, researchers are able to fine tune the hardware performance to directly match their experimental needs. Unfortunately, this is not always the case and researchers are usually forced to either tweak their experimental design or are required to build "in-house" tools to conduct their experiments. With this in mind, our goal was to develop instrumentation and software for cell-by-cell recording of Ca^{++} transients that is easily integrated into current laboratory setups. This module (the Calcium Transient Image Cytometer, or CTIC) will interface easily to commercially available high content microscopy workstations, which already perform plate scanning

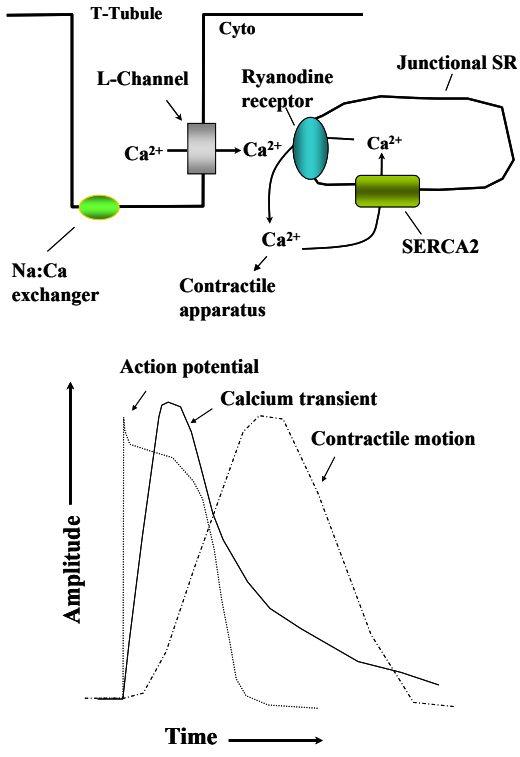

Figure 1. The cardiac contractile cycle[1] **(adapted from Bers 2002).**A) Calcium-induced calcium release from the SR and the role of SERCA2 to reuptake Ca^{++} into the SR. B) The cardiac action potential, calcium transient and myocyte contraction.

and image acquisition, to enable video burst acquisition and analysis of calcium transients in a fully automated (high throughput screening) mode. The CTIC will electrically stimulate (or pace) the cells, record the resulting Ca^{++}transients from cells in microtiter plates (e.g., with 96 wells), and automatically quantify characteristics such as the duration of the Ca^{++}waves on a cell-by-cell basis in a fully automated manner on large scale screens (e.g., tens to hundreds of thousands of compounds).

EXPERIMENTAL DESIGN AND METHODS

For the first phase of our development, we interfaced the CTIC module to our high content screening system, the EIDAQ 100 (Q3DM, San Diego, CA). The EIDAQ 100 is functionally identical to (i.e., utilizes the exact same hardware and controlling software, CytoShop, as) the later-released Beckman Coulter IC 100. Q3DM sold the EIDAQ technology to Beckman Coulter in December 2003 for manufacturing and worldwide distribution.

The EIDAQ 100 includes: 1) an inverted epifluorescence microscope (Nikon Eclipse TE2000-U), 2) an intensity- feedback stabilized 100 W Hg arc lamp developed by Q3DM, 3) excitation and emission filter wheels, 4) a motorized stage with XY-axes control, and 5) a piezoelectric *Z*-axis control for fast, precise autofocus. The emission light path on the EIDAQ 100 is split by a Nikon multimode module to the autofocus and scientific grade CCD cameras. The EIDAQ 100's scientific grade camera was removed from the optical path but left powered on and connected to allow normal CytoShop operation.

Biological considerations. We developed the CTIC instrument utilizing cultured NRVMs, which can be prepared quickly (as opposed to the more lengthy culturing required for ESCMs). The cultured NRVMs respond to electrical stimulation a Ca^{++} transient accompanied by a contractile-twitch. The contractile calcium transients are transient elevations in intracellular calcium, which can be tracked via use of intracellular fluorescent calcium indicators (e.g., Fluo-3 and Fluo-4) and the digital fluorescence microscopy technology as represented by the EIDAQ 100 and CTIC hardware module. An instrument designed to electrically stimulate, record, and quantify contractile calcium transients from NRVMs cultured in 96-well dishes, will be capable of recording electrically stimulated contractile calcium transients in cultures of embryonic stem cells to provide a definitive readout of the stage of differentiation towards cardiomyocytes. That is, we hypothesize that different stages of differentiation will be characterized by measurement differences in the Ca^{++} transients of these beating cells. The instrument will also likely be useful for testing compounds on NRVMs (or cardiac myocytes from other sources) in a high throughput manner for potential negative or positive inotropic effects, or for arrhythmic effects.

The NRVMs were cultured in 96-well dishes (Nunc coverslip-glass-bottom plates) pretreated with 1% gelatin that was cross linked with glutaraldehyde to improve cell adherence.

Sensitivity to intracellular calcium kinetics is a fundamental requirement for the CTIC module because the goal of the instrument is to distinguish healthy contractile ESCMs from background non-contractile stem cells. To test this sensitivity, contractile calcium transients were quantified in two populations of NRVMs: NRVMs exposed to control media vs. media supplemented with 100-nM thyroid hormone (T3), which is known to increase the expression of SERCA2, leading to more rapid calcium re-uptake, and, overall accelerated kinetics of the contractile calcium transients (Figure 2). Thus, for all NRVMs preparations, half of the wells contained control medium (DMEM:F12 with 0.25% FBS) and the remainder included the same media supplemented with T3. The cells were maintained 48 to 72 hrs prior to scanning. On the day of scanning, the cells were incubated with a 3 μM solution of the acetyoxymethylester form of an intracellular calcium indicator (either Fluo-4 AM or Fluo-3 AM) in the base media for 25 minutes. The plasma membrane is permeable to the acetoxymethylester forms of Fluo-3 and Fluo-4, which are modified intracellularly by esterases to an impermeable form. The loading solution was also supplemented with 200 ng/ml Hoechst 33342 to label DNA within the nuclei. This concentration of Hoechst provides adequate label of the nuclei and was non-toxic under these conditions. Thus, the labeling procedure introduced a green-fluorescence channel fluorescent calcium indicator into the cytoplasm of the NRVMs (Fluo-4) and a blue-fluorescence channel nuclear stain (Hoechst). The nuclear channel was used for autofocus at each well; additionally, images obtained of the nuclei provided information regarding cellular position that was helpful in assigning boundaries between cells in the segmentation procedure applied to the green-channel images. Following loading, cells were placed in 150 μl of HEPES-buffered physiological saline (Tyrodes solution) supplemented with 5-mM glucose and 2-mM calcium chloride.

CTIC Instrumentation Module. The components of the CTIC module include a separate PC running Windows XP and control software programmed in Labview 8.0, (National Instruments, Austin, TX), a National Instruments data acquisition I/O board, the NI-PCI-6251, a stimulator/electrode assembly (lowered and raised using a computer-controlled Sutter Instruments (Novato, CA) micromanipulator, the MP-285), a Grass Technologies (West

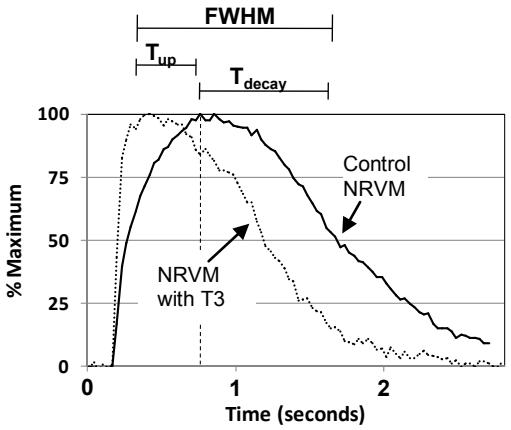

Figure 2. Parameters for quantification of contractile calcium kinetics are shown. The transients are for a control NRVM (solid line) and for a NRVM maintained in 100-nM thyroid hormone (dotted line) normalized to their minimum (0%) and maximum values (100%). The kinetic parameters derived from the transients are the Full Width at Half Max (FWHM) which is the time required for progression from the 50% point on the upstroke to 50% point on the downstroke, T_{up}, which is the time from the 50% point to 100% on the upstroke, and T_{decay}, which is the time period from the 100% point to the 50% point on the decay phase.

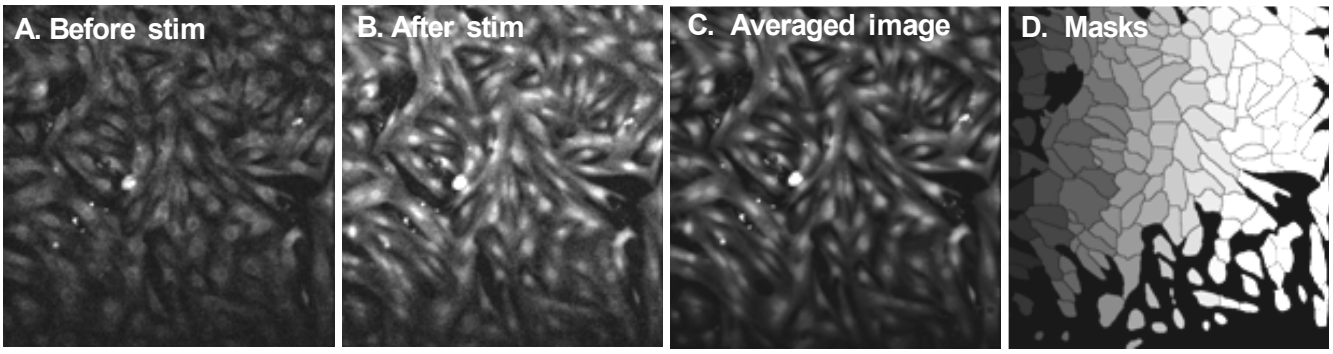

Figure 3. **Strategy for segmentation of images derived from Flou-4 loaded cardiac myocytes** A: A raw image of NRVMs prior to stimulation (low Ca^{++}, dim Fluo-4) is shown. B: The same field of view is shown immediately after electrical stimulation (high Ca^{++}. bright Fluo-4). C: The average of all images in the video stream. D: The masks segmented from the averaged image with each cell mask labeled by a unique gray-scale intensity.

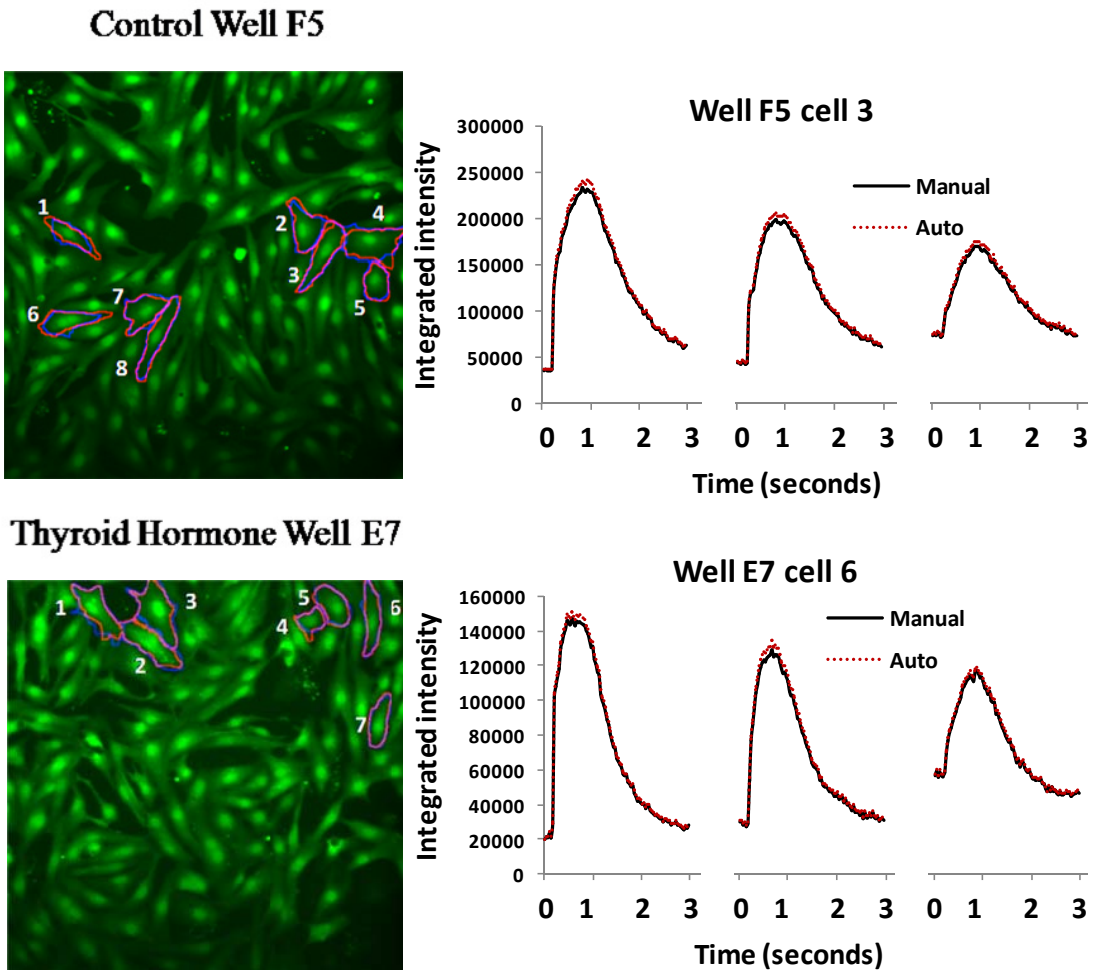

Figure 4. Manual vs. automated segmentation of images obtained from fluo-4 loaded NRVMs.*Upper,* Cell masks drawn manually (red) or via automatic segmentation (blue) are shown for cells cultured in control media; calcium transients are shown for cell #3 which was stimulated 3 times at 10 min intervals. *Lower,* Cell masks for cells cultured for 72 hr in 100 nM thyroid hormone; calcium transients are shown for cell #6.

Warwick, RI) S48 square pulse stimulator, and a high speed scientific-grade CCD camera. Back- and front-illuminated EMCCD cameras were tested including the iXon DU-897 from Andor (South Windsor, CT), the ImageEM C9100-13 from Hamamatsu (Hamamatsu, Japan), and the QuantEM from Roper/Photometrics (Tuscon, AZ).

Data Acquisition. NVRM green channel video streams of 3-10 seconds at 30fps, comprising 1-3 electrical stimuli and triggered by

the electrical signal to the arc lamp shutter on the EIDAQ 100, were collected and stored directly to the hard drive of the PC controlling the CTIC module. Prior to video stream acquisition, the EIDAQ 100 auto-focused on the nuclear channel of the NVRMs. All images were captured at a magnification of 20x 0.50 numerical aperture (NA) and a 1x intermediate camera zoom.

Cytometric Analysis of Time-Series Images. Image analysis was carried out to obtain cytometric data by using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA), Matlab (The MathWorks, Inc., Natick, MA) and CellProfiler (BROAD Institute, Cambrigde, MA). Image analysis featured averaging of images in the time-series, background subtraction, and automatic segmentation.

An average of the time-series green-fluorescence channel images was corrected for background using ImageJs "Rolling Ball" background subtraction algorithm [7]. This background corrected image was then automatically segmented using CellProfilers "IdentifyPrimaryAutomatic" algorithm [8], which performs watershed with local maxima as the tessellation centers (Figure 3). This automated segmentation typically resulted in very good agreement with cells segmented manually (Figure 4). The resultant cytoplasmic masks were then applied to each time frame image to quantify single cell integrated fluorescence intensity as shown in the Figure 4 graphs.

RESULTS AND CONCLUSIONS

We report here the development of monitoring system capable of automatically recording and analyzing electrically stimulated calcium transients from cells exhibiting the phenotype of cardiac myocytes. Integration of the CTIC with the EIDAQ 100 was successfully achieved, and approximately 1300 video clips were obtained from electrically stimulated NRVMs. Cells cultured in thyroid hormone for 72 hr displayed markedly accelerated contractile calcium transients, as quantified by the automated methodology. For example, in a typical data set derived from over 2000 cells, T_{up}, T_{decay}, and FWHM were reduced by 73%, 56%, and 64%, respectively, by thyroid hormone (Figure 5, data set #1). Furthermore, similar results were obtained for two additional experiments, performed on different preparations of NRVMs (Figure 5, data sets #2 and #3), which further illustrates the reproducibility of the effect of thyroid hormone on the NRVMs, and the consistency of the automated methodology..

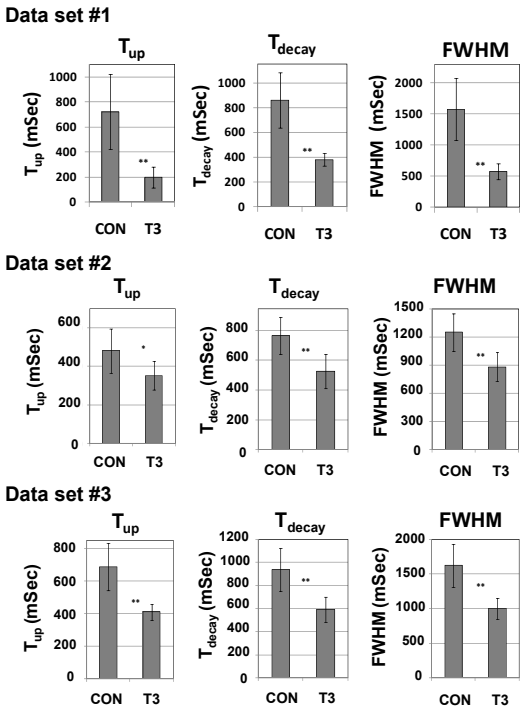

Figure 5. Effect of thyroid hormone (T3) on contractile calcium kinetics in NRVMs measured by automated microscopy and image analysis. Each bar represents the mean ± SD for n= 8 wells for control cells or n= 12 to 22 wells for T3-treated cells. An average of 110 cells were measured, per well.The experiment was repeated with 3 different preparations of NRVMs which were analyzed independently (data sets 1, 2, and 3).

Thus, the system can detect and quantify the effect of test compounds on the contractile phenotype of cells cultured under conditions very similar to those utilized in experiments designed to produce hESC-CMs. The apparatus could also be utilized to test for potential toxic or beneficial effects on fully differentiated cardiac myocytes, which may have applications for pharmaceutical screening and lead-compound characterization.

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